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TITLE: **A Novel Association and Therapeutic Targeting of Neuropilin-1 and MUC1 in Pancreatic Cancer**

PRINCIPAL INVESTIGATOR: **Pinku Mukherjee**

CONTRACTING ORGANIZATION: **University of North Carolina at Charlotte
Charlotte, NC 28223-0001**

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14. ABSTRACT <p>We hypothesized that MUC1, a transmembrane glycoprotein that is overexpressed in >80% of pancreatic ductal adenocarcinoma (PDA) induces a pro-angiogenic tumor microenvironment by increasing the level of NRP1 and VEGF thereby enhancing angiogenesis and metastasis. We report that MUC1hi PC cells and tumors in vitro and in vivo not only express higher levels of NRP1 but also express higher levels of VEGFR2 and its phosphorylation forms as well as secrete higher levels of VEGF than MUC1low PC cells. This enables the MUC1hi/NRP1hi cells to induce endothelial cell tube formation and generate long ectopic blood vessels and enhanced distant metastasis. In the proposal, we also hypothesized that blocking the interaction between VEGF165 and NRP1 within the tumor microenvironment will lead to therapeutic benefit. Indeed, in vivo blocking NRP1 significantly reduces tumor burden in the MUC1hi mouse and human tumors. For the in vivo MUC1-specific tumor targeting, we demonstrate that the antibody TAB004 binds MUC1+ve tumors in vitro and in vivo by Immunohistochemistry of primary human PDA tissues and by live animal imaging. Thus, NRP1 is a promising target for MUC1hi PDA. We conjugated TAB 004 to the a specific NRP-inhibitor for in vivo targeted therapy and show higher efficacy of the conjugate as compared to TAB004 alone or NRP-inhibitor alone in reducing VEGF secretion.</p>					
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Introduction:

Pancreatic cancer (PC) is a lethal disease. We hypothesize that MUC1 induces a pro-angiogenic tumor microenvironment by increasing the level of NRP1, thus enhancing angiogenesis, disease progression and metastases. Second, blocking the interaction between VEGF₁₆₅ and NRP1 within the tumor microenvironment will lead to therapeutic benefit. We report progress for all three tasks that we had proposed. I have thus broken the report down by tasks. The objective and specific aims are stated below. This is followed by the individual tasks with the progress made for that task. **We show 16 figures in total. Figures 1-8 are for Task 1, Figures 9-11 are for Task 2, and Figures 12-16 are for Task 3.**

Objectives/Specific Aims:

To test the effects of MUC1+ve PC cells on *in vitro* endothelial cell function in a NRP1-dependent fashion.

To determine if MUC1 up-regulates NRP1 and creates a pro-angiogenic niche *in vivo*.

To directly target angiogenesis within the tumor microenvironment by using TAB004 MUC1 antibody conjugated to a peptide blocking VEGF-NRP1 binding.

Keywords

Neuropilin-1, MUC1, VEGF, Angiogenesis, Pancreatic Cancer

Overall Project Summary

Task 1:

To test the effects of MUC1+ PC cells on *in vitro* endothelial cell function in an NRP1-dependent fashion (0-9 months). For this task we perform *in vitro* angiogenesis assays to assess the ability of MUC1-expressing pancreatic cells to enhance endothelial cell proliferation, invasion and tube formation via NRP1.

We first showed that MUC1 and NRP1 were expressed in the primary human PDA, but minimally in the normal pancreas (**Figure 1A**). We then determined if a correlation exists between MUC1 and NRP1 in a panel of human PDA cell lines that endogenously express high or low MUC1. Next we determined if MUC1 regulates NRP1 expression by conducting gain of function (stably transfected full length MUC1 in MUC1-low cells) and loss of function studies (by knocking down MUC1 using MUC1 specific siRNA). **Figure 1B** clearly shows that cells expressing high endogenous MUC1 also have high NRP1 while cells with low endogenous MUC1 has low NRP1 with exception (Panc1). The correlation between MUC1 and VEGFR2 levels were not consistent among the cell lines. We showed that overexpressing full length MUC1 in two separate MUC1low cell lines (BxPC3 and Panc02) can induce higher expression of NRP1 (**Figure 1C**) while knocking down MUC1 from three other MUC1hi cell lines (HPAC, CFPAC and HPAFII) can reduce NRP1 expression (**Figure 1D**). Whether this regulation is direct or indirect is not yet delineated.

We have established two mouse PDA cell lines, i.e. KC and KCKO, from spontaneously arising PDA tumors in WT (Muc1 intact) versus in Muc1 null mice [1]. A gene microarray was conducted in those two cells. Among those selected genes, KC cells showed higher NRP1 and NRP2 levels (Figure 2). Different from NRP2, NRP1 is mainly expressed on vascular endothelial cells and involved in the VEGF-induced angiogenesis. To validate the NRP1 expression data

obtained from gene microarray, we further confirmed the expression of Muc1 in KC cells by flow cytometry (Figure 3A, left panel), and found KC cells displayed moderately higher NRP1 on their surface than KCKO cells (Figure 3A, right panel). Data from Western blotting showed consistent results (Figure 3B). To further elucidate the mechanism, we determined the other VEGF receptor levels in these cells. This is especially important as NRP1 is only a co-receptor of VEGF and signaling through VEGFR2 is critical for the angiogenic signaling to occur [2]. We show that KCKO (Muc1 null) cells have moderately lower levels of VEGF receptor 1, 2, and 3 than its counterpart KC cells which have an intact Muc1 (VEGFR2 in Figure 3B, and data not shown).

Then, to elucidate the role of higher levels of NRP1 and VEGFR2 in KC cells, we compared the cell response to VEGF stimulation. Due to the difficulty of detecting phosphorylation of VEGFR2 *in vitro* (data not shown), Erk activation was evaluated instead which is downstream of VEGFR2 activation. There was higher level of endogenous Erk phosphorylation in KC cells (Figure 4A), and 60 min after stimulation with VEGF there was an increase of p-Erk in KC cells but not in KCKO cells. Erk is involved in many aspects in response to VEGF, including proliferation, survival, EMT, and angiogenesis. Thus, the cell proliferation was compared between KC and KCKO cells. It showed in Figure 4B, both cells proliferated similarly (left panel). VEGF did not promote cell proliferation, and blocking NRP1 function by its antagonist peptide A7R did not prevent KC cells from proliferation (right panel), which suggested that VEGF signaling was not required for KC or KCKO cell proliferation. However, the survival of Muc1-bearing KC cells was largely better than Muc1-null KCKO cells in response to serum starvation (Figure 4C). Whether the poor survival of KCKO cells is due to Muc1 deficiency or lower level of Muc1-associated VEGFR2 signaling will be clarified. Additionally, we observed that the Muc1/MUC1-bearing KC or BxPC3 cells migrated slightly better than their counterparts in the scratch assay (Figure 5).

Furthermore, KC cells secreted significantly higher level of VEGF than KCKO cells (Figure 6A), which could be partially inhibited by A7R (Figure 6C) without clearly affecting cell numbers (Figure 6B). These data suggest that VEGF secreted by tumor cells can positively feedback themselves through the NRP1-VEGFR2 signaling.

Lastly, we studied whether the supernatants from KC and KCKO cell cultures can differentially regulate the NRP1 level and its associated function in murine endothelial cells, 2H11. As shown in Figure 7A, 2H11 cells endogenously expressed high levels of NRP1 (left and middle panels), which made it difficult to be further modulated by tumor-conditioned supernatants (right panel). We did not test the other VEGF receptors and are planning to conduct those analyses in the future. Other endothelial cell lines will also be tested similarly. Even though it might not affect NRP1 levels, it is possible that there will be differential angiogenic/oncogenic signaling. Here, we utilized an *in vitro* endothelial cell tube formation assay to evaluate the angiogenesis induction. The supernatants from KC cell cultures dramatically induced tube formation in 2H11 cells (Figure 7B), which was suppressed by blocking NRP1 activity or neutralizing VEGF (Figure 7C). Combination of A7R with anti-VEGF antibody had additive effect in significantly inhibiting the tubular structure formation on the matrigel basement. In addition, direct pre-treatment of KC cells with A7R effectively decreased VEGF secretion in the supernatant (Figure 8A). The supernatant from A7R pretreated KC cells induced significantly less tube formation (Figure 8B).

Figure 1: MUC1 up-regulates NRP1 expression in pancreatic cancer cells. **A)** Expression of MUC1 and NRP1 in human pancreas tissues. Left, representative images; right, quantitation of protein expression. **B)** CFPAC, HPAFII, and HPAC express moderate/high levels of MUC1. These MUC1-hi cells all express moderate to high levels of NRP1. Capan2, Panc1, Capan1, HS766T, and Miapaca2 cells have low/intermediate levels of MUC1. Other than Panc1, these low MUC1 cells also express low levels of NRP1. **C)** When BxPC3 and Panc02 cells were stably transfected with full length MUC1, NRP1 expression was substantially increased. **D)** Similarly, when MUC1 was down regulated in HPAC, CFPAC and HPAFII cells using siRNA, NRP1 levels were significantly downregulated. β -actin served as control for equal loading of protein.

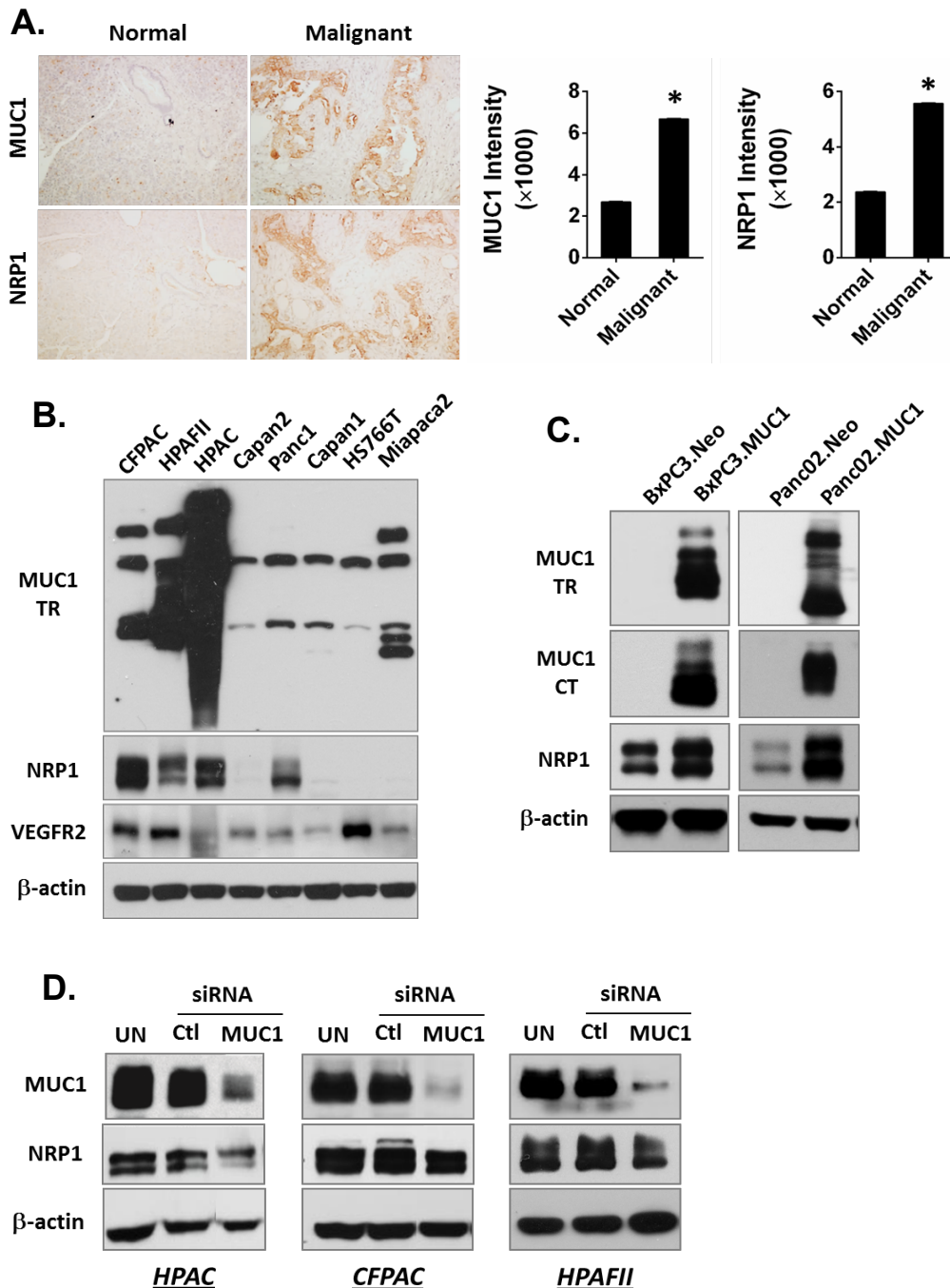


Figure 2: Microarray profile for selected gene expression in KC versus KCKO *in vitro*. RNA from KC and KCKO cells were extracted and run for gene microarray. Selected genes expression out of pre-chosen 323 genes was shown. Those 323 genes were pre-chosen based on that fold change of gene expression in KC versus KCKO is ≥ 3 , or ≤ -4 .

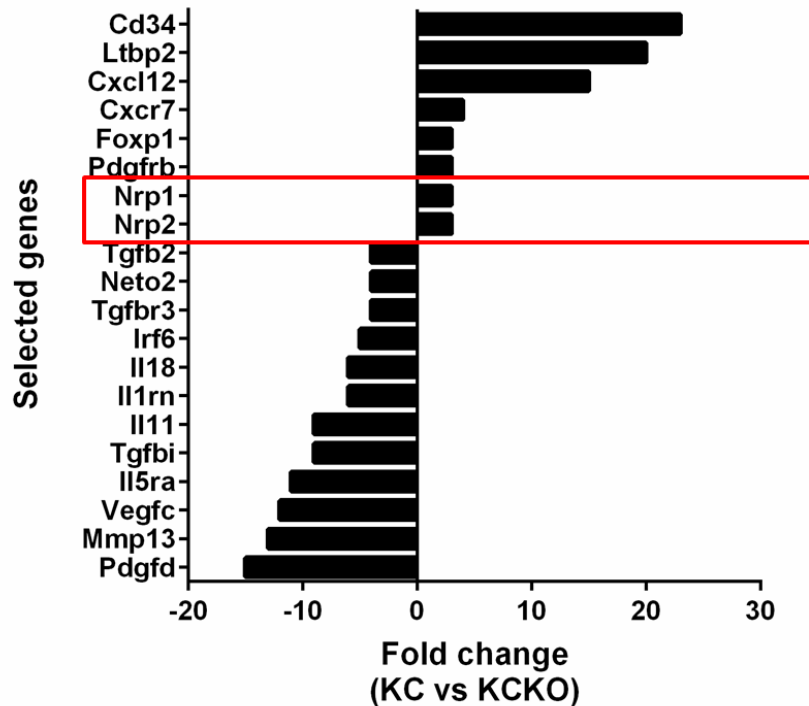


Figure 3: Higher Muc1 expression is associated with higher NRP1 level in spontaneously arising mouse PDA cells. A) KC cells are positive for MUC1 and express higher NRP1 *in vitro*, determined by flow cytometry analysis. **B)** The expression of MUC1, NRP1, and VEGFR2 are confirmed in cell lysates by Western Blot.

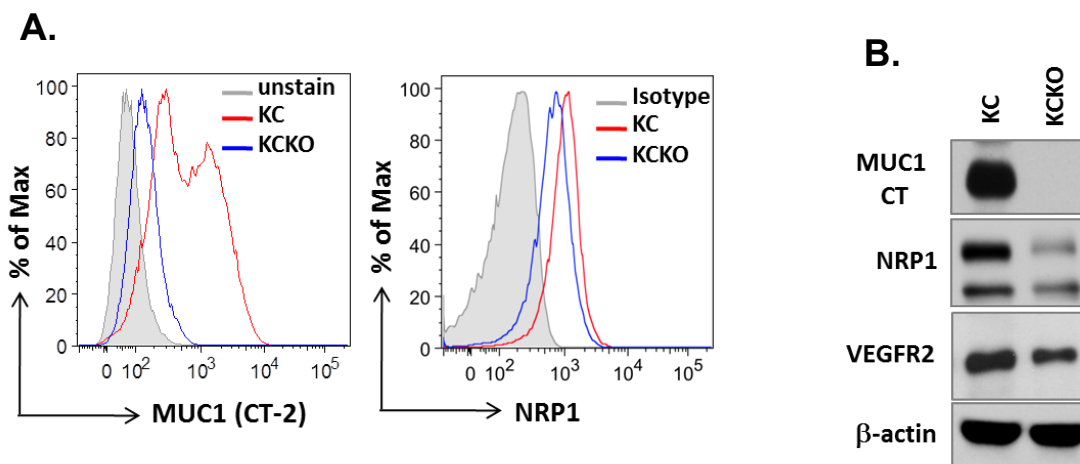


Figure 4: VEGF signaling is not required for KC or KCKO cell proliferation but may be critical for cell survival. **A)** KC cells have higher endogenous level of phospho-Erk and respond better to VEGF stimulation. **B)** KC and KCKO cells have similar proliferation rate (Left panel), which is not significantly changed by VEGF stimulation or NRP1 blockade (Right panel). **C)** KC cells survive better in response to serum starvation.

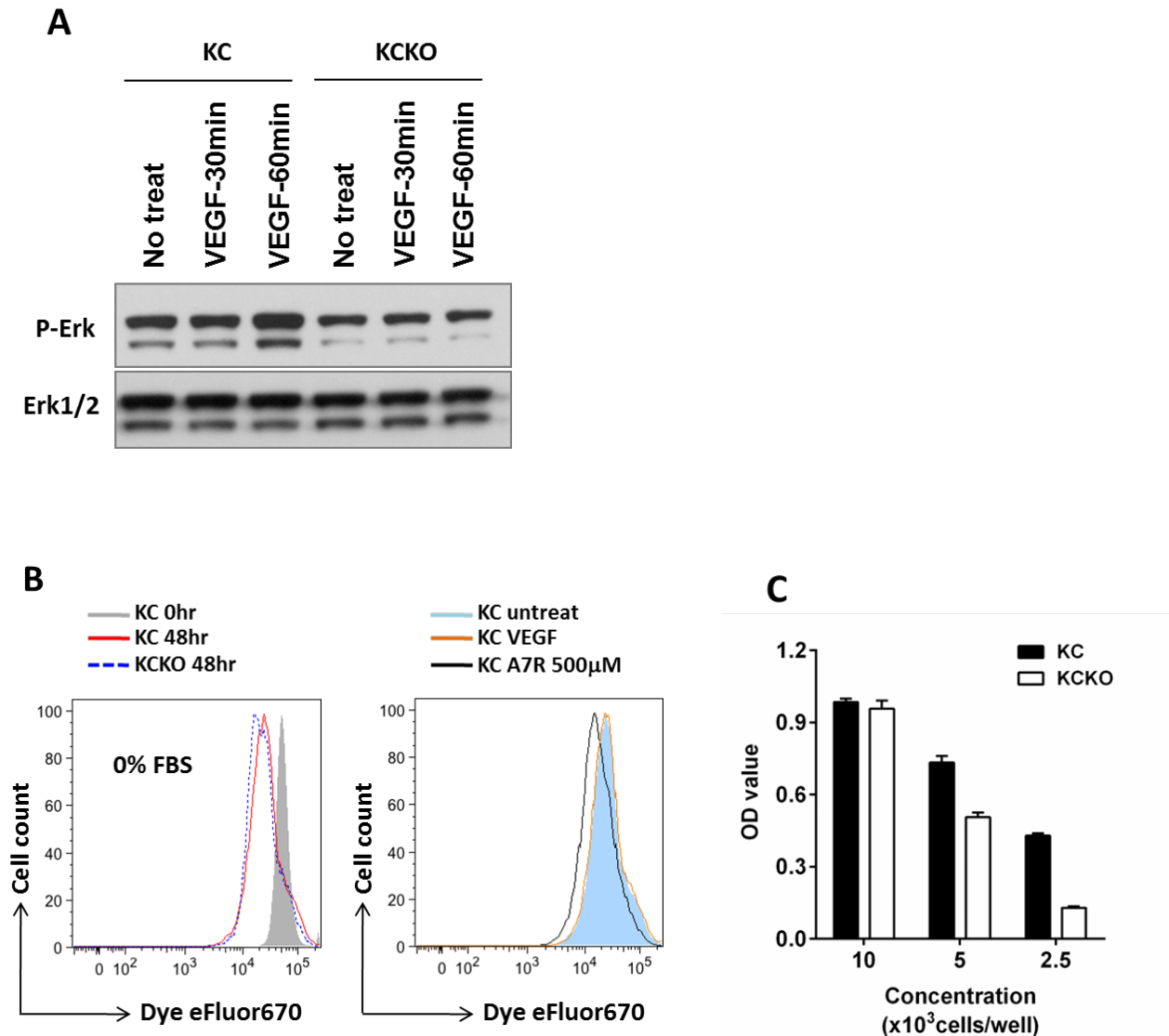


Figure 5: MUC1 enhances cell migration *in vitro*. Cells are plated and scratched. The closure of gap was monitored in **A)** KCKO versus KC and **B)** BxPC3.Neo versus BxPC3.MUC1.

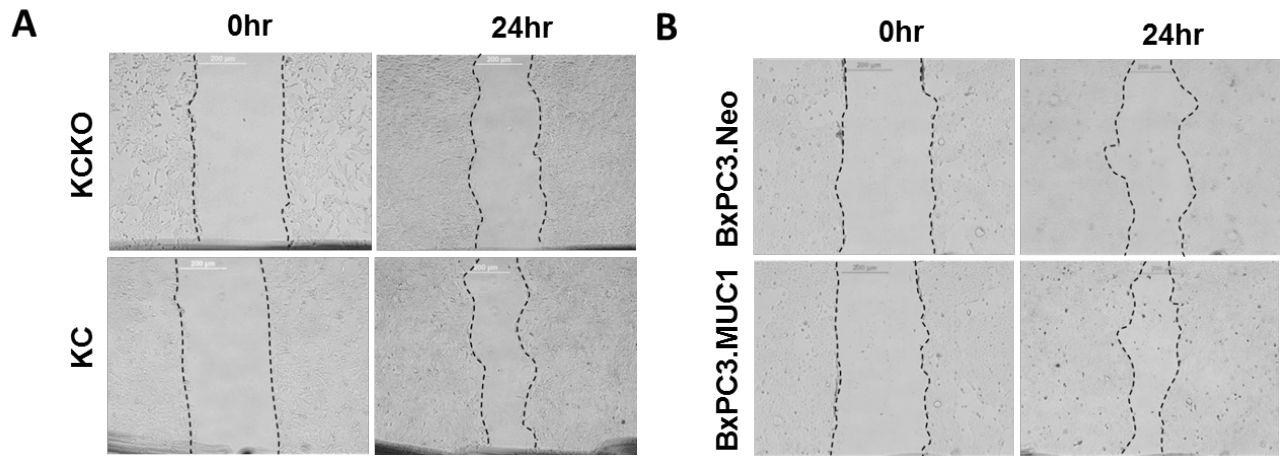


Figure 6: Blockade of NRP1 signaling decreases VEGF production by KC cells. **A)** KC cells produce more VEGF. **B)** NRP1 antagonist A7R does not significantly affect KC cell viability. **C)** A7R decreases VEGF secretion from KC cells.

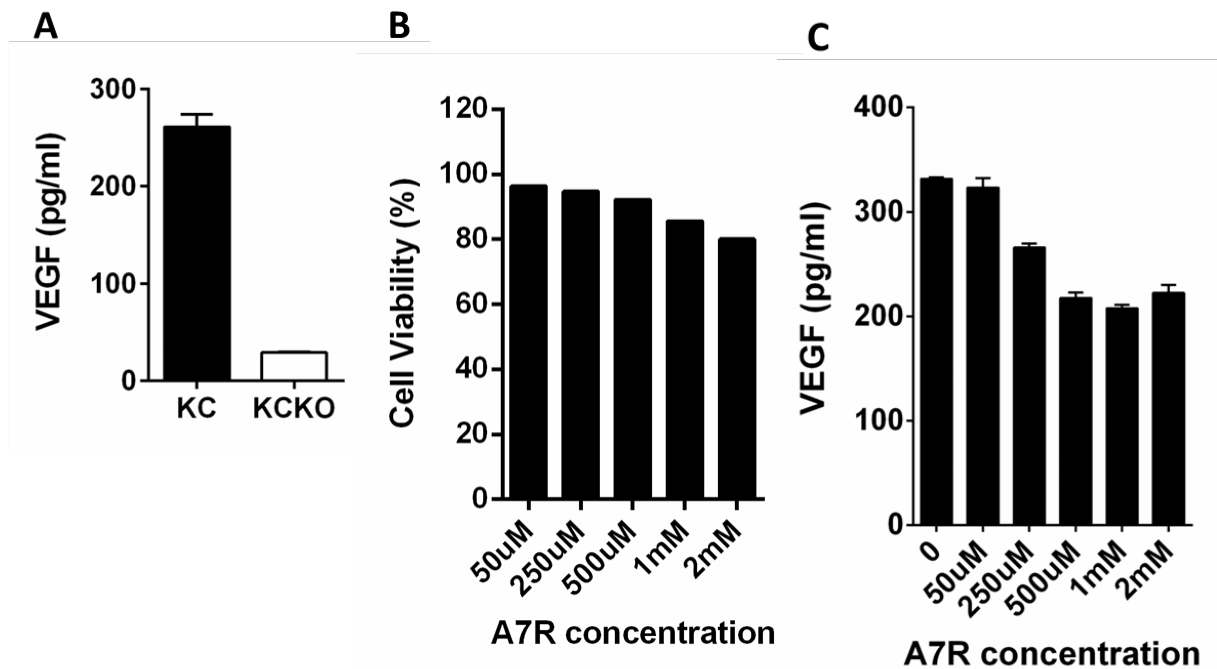


Figure 7: KC culture supernatant promotes tube formations in 2H11 endothelial cells and can be reversed by blocking VEGF signaling. **A)** 2H11 cells express NRP1 and VEGFR2, determined by flow cytometry (left panel) and Western Blot (middle panel). The NRP1 level is not changed by tumor conditioned medium (right panel). **B)** Tumor-derived conditioned medium from KC cell culture promotes more tube formation in 2H11 cells. **C)** The tube formation induced by KC culture medium can be reversed by NRP1 antagonism and VEGF neutralization. Left, representative images; right, quantitation for tube formation.

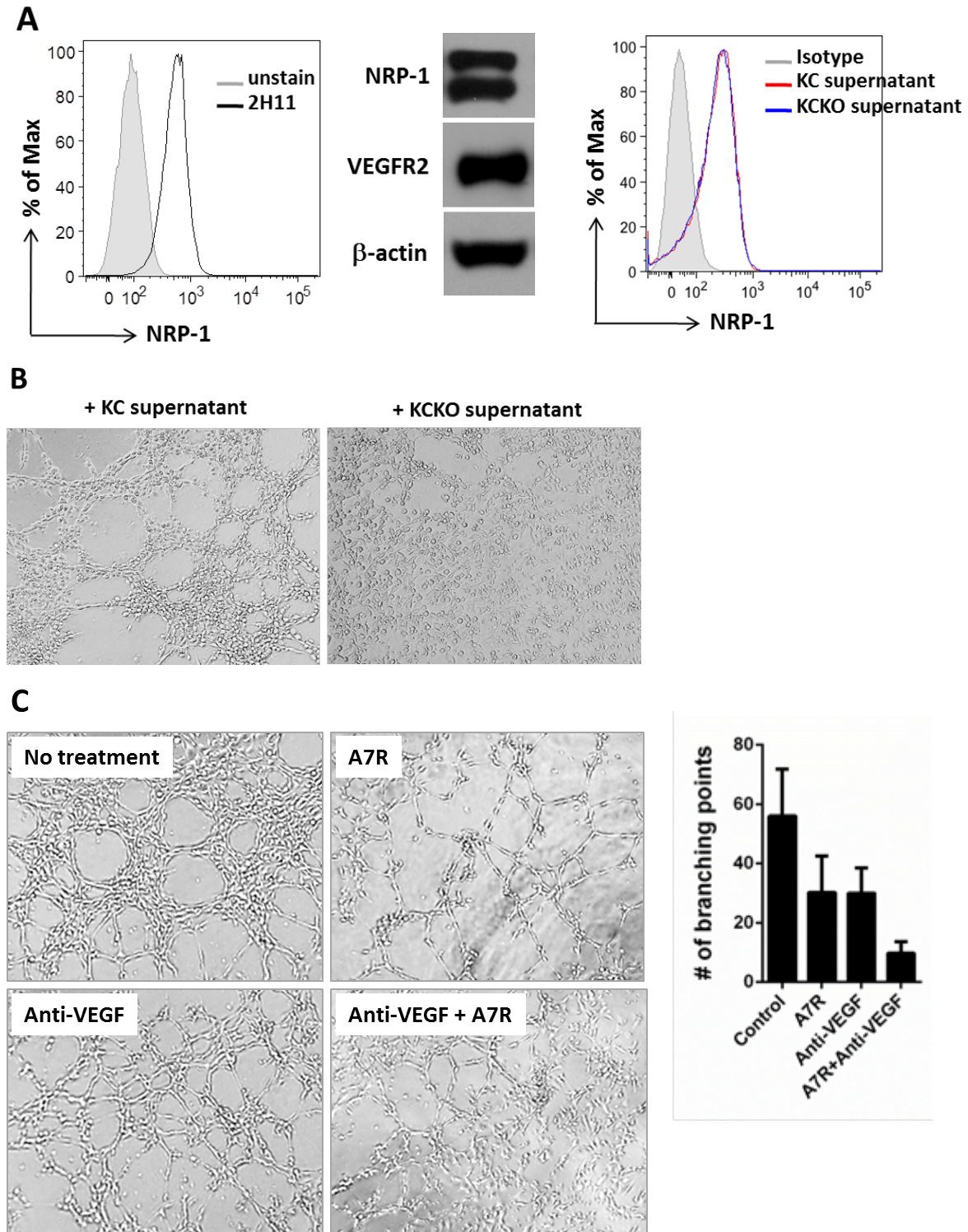
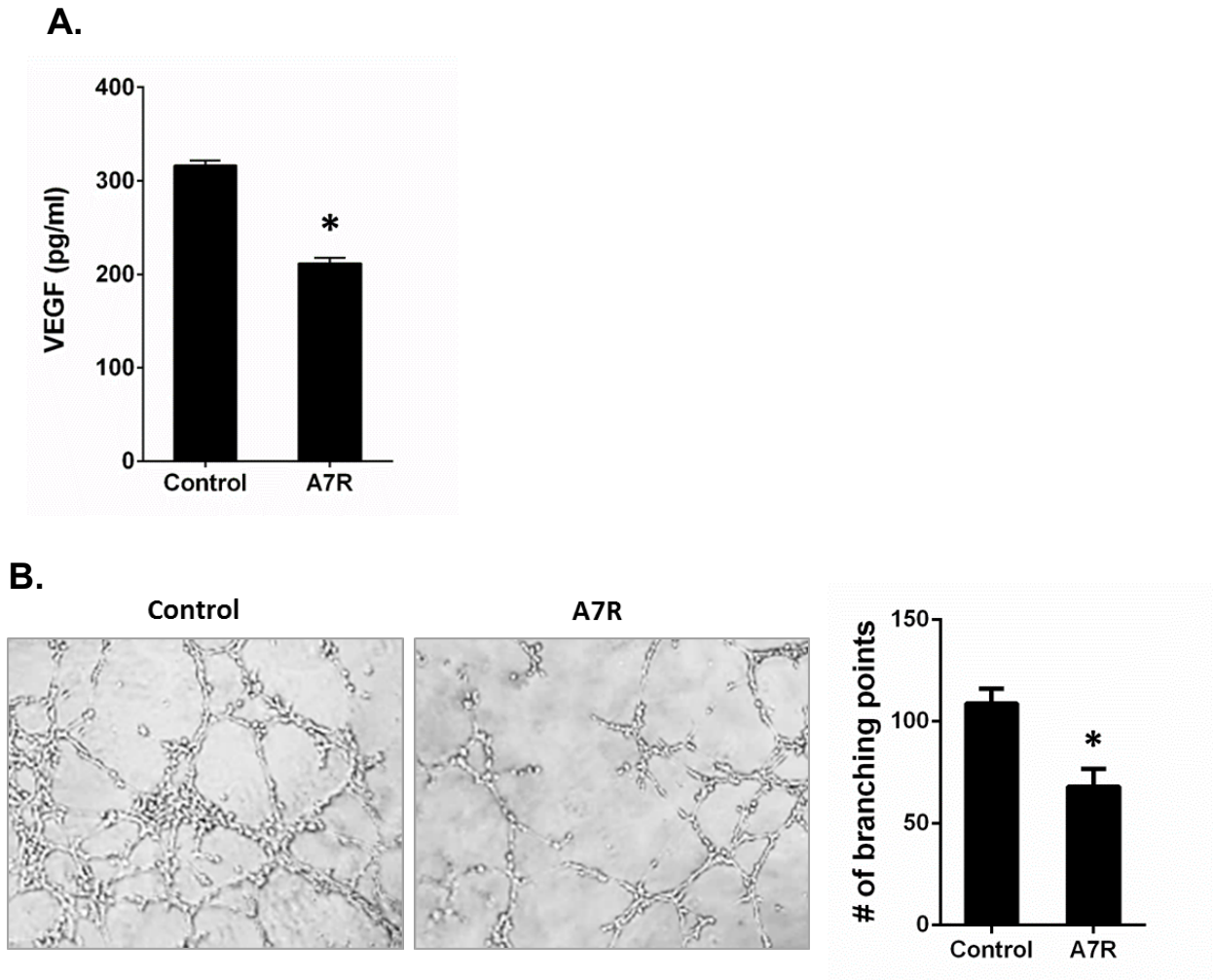


Figure 8: Direct blocking of NRP1 on KC cells partially reduces the endothelial tube formation. **A)** A7R-pretreated KC cells produce less VEGF. **B)** Conditioned medium from A7R-pretreated KC cell culture induce less tube formation in 2H11 cells. Left, representative images; right, quantitation for tube formation.



Task 2:

To determine if MUC1 up-regulates NRP1 and creates a pro-angiogenic niche *in vivo* (0-22 months). For Task 2, we will use our mouse models of pancreatic cancer to determine if MUC1-expressing pancreatic tumors have enhanced intra-tumoral levels of NRP1 and angiogenesis.

KC and KCKO cells were subcutaneously injected into mouse to evaluate the activation of VEGFR2 signaling and pathways that are associated with angiogenesis. VEGFR2, the major positive signal transducer for both physiological and pathological angiogenesis is selectively expressed on vascular endothelial cells. The binding of VEGF to its receptors induces dimerization and subsequent receptor phosphorylation, which then leads to the activation of several intracellular downstream signaling pathways promoting angiogenesis [3]. Thus we looked at the levels of VEGFR2 phosphorylation *in vivo* in KC and KCKO tumors. Data were summarized as [Figure 9](#). It was of great interest to demonstrate that not only were the levels of NRP1 and VEGFR2 significantly lower in the KCKO tumors, the phosphorylation at tyrosine sites of 1175, and 996 were significantly low ([Figure 9](#)), which is of course because the receptor level itself was low. Meanwhile, the higher levels of N-Cadherin and Vimentin and lower level of E-Cadherin were observed in KC tumors ([Figure 9](#)), which favor the EMT transition [4]. Together, this data suggested that in KCKO tumors, lack of Muc1 regulated NRP1 and VEGFR2 and thereby downregulated the angiogenic signaling. This is significant since NRP1 binds VEGFA₁₆₅ and only when co-expressed with VEGFR2, it enhances the binding of VEGFA₁₆₅ to VEGFR2 by 4- to 6-fold and results in sustained vascular permeability, inflammation, and endothelial cell migration. The question still remains whether the low receptors are due to low endothelial cells and low vessels, or due to the tumor cells themselves as NRP1 and VEGFR are expressed on both the epithelial and the endothelial cells.

To further demonstrated that MUC1 up-regulates NRP1 and creates a pro-angiogenic niche *in vivo*, we did tissue immunohistochemistry staining for the angiogenesis-related proteins. In [Figure 10](#), data showed that expression of NRP1, VEGF, CD31, and proliferating cell nuclear antigen (PCNA) were higher in the spontaneously arising KC tumors than those in Muc1 null KCKO tumors. This data strongly suggests that there is more onset of angiogenesis when Muc1 is present.

Based on the above findings and before we started the MUC1-specific tumor target therapy, we first validated the activity of NRP1 antagonist A7R in KC tumor-bearing mice. KC cells were subcutaneously injected into immune-competent C57BL/6 mice and after 4-5 days after cell inoculation, those mice were s.c. treated with A7R, 3 times a week. The growth of KC tumor was significantly reduced over the time ([Figure 11A](#)). Further, BxPC3.MUC1 tumor bearing mice were also treated with the NRP1 blocking peptide A7R. Two weeks after cell injection, we randomized groups and started therapy with A7R for 5 weeks, PBS served as the vehicle control. Mice bearing BxPC3.MUC1 tumors responded well to the mono-therapy with significantly lower tumor burden between treated and untreated mice and with 4 out of 7 mice showing a complete response ([Figure 11B](#)). Mechanistically, Western blot data from treated KC tumors further confirmed the blocking activity of A7R on the NRP1 and VEGFR2 activities ([Figure 11C](#)).

Figure 9: MUC1 enhances VEGF signaling and promotes EMT. The VEGFR2 activation and EMT switch are assessed in tumor lysates from the respective tumor bearing mice. KC and KCKO cells are subcutaneously injected into C57BL/6 mice. Twenty-six days after cell injection, tumors are retrieved and the indicated proteins in lysates are analyzed by Western Blot. Left, representative images; right, normalized protein expression level.

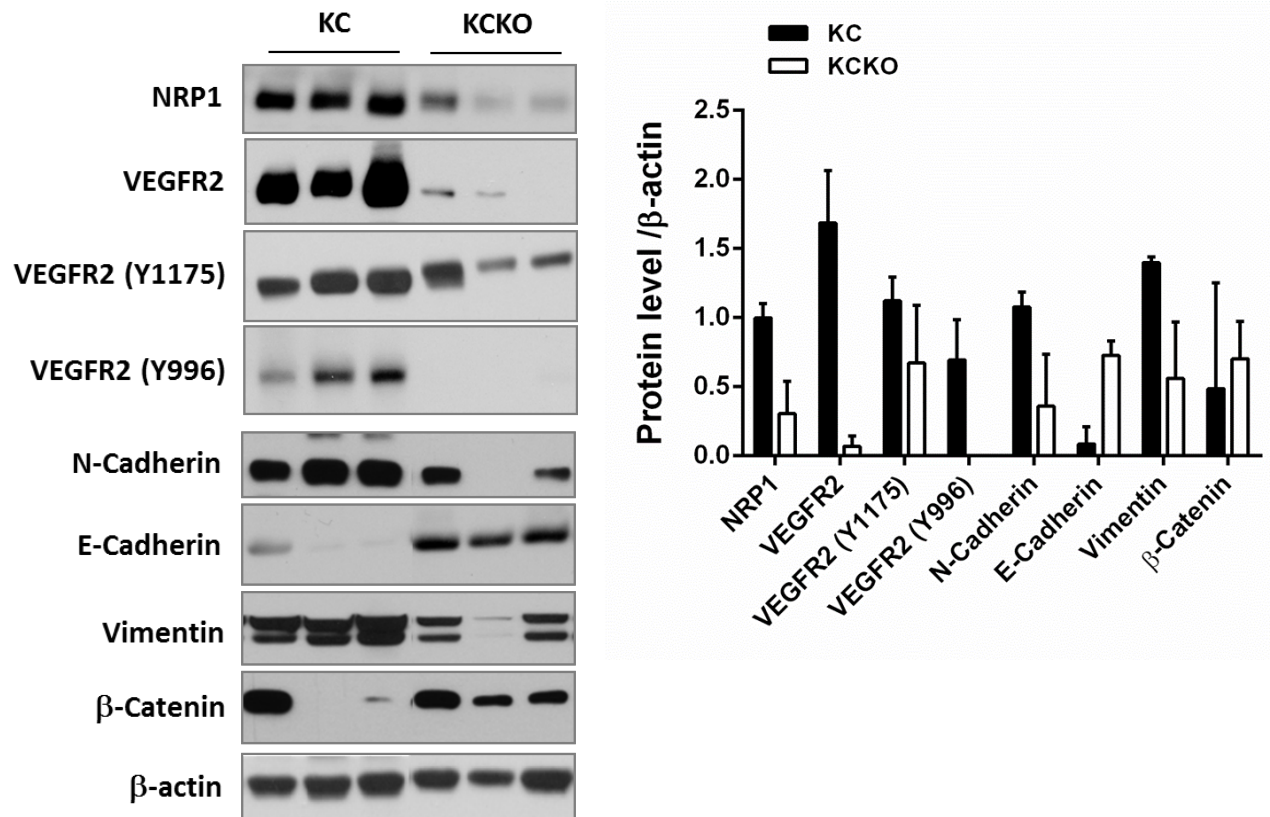


Figure 10: Higher expression of angiogenesis-associated proteins in spontaneously developed KC compared to KCKO tumors. Representative images of immunohistochemistry staining (IHC) for the angiogenesis-associated proteins.

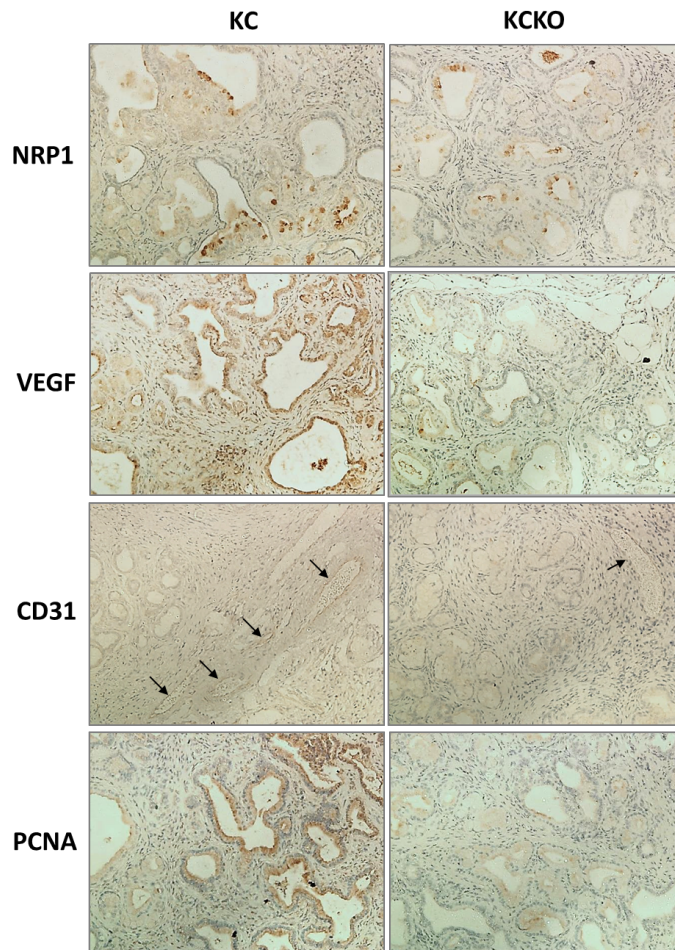
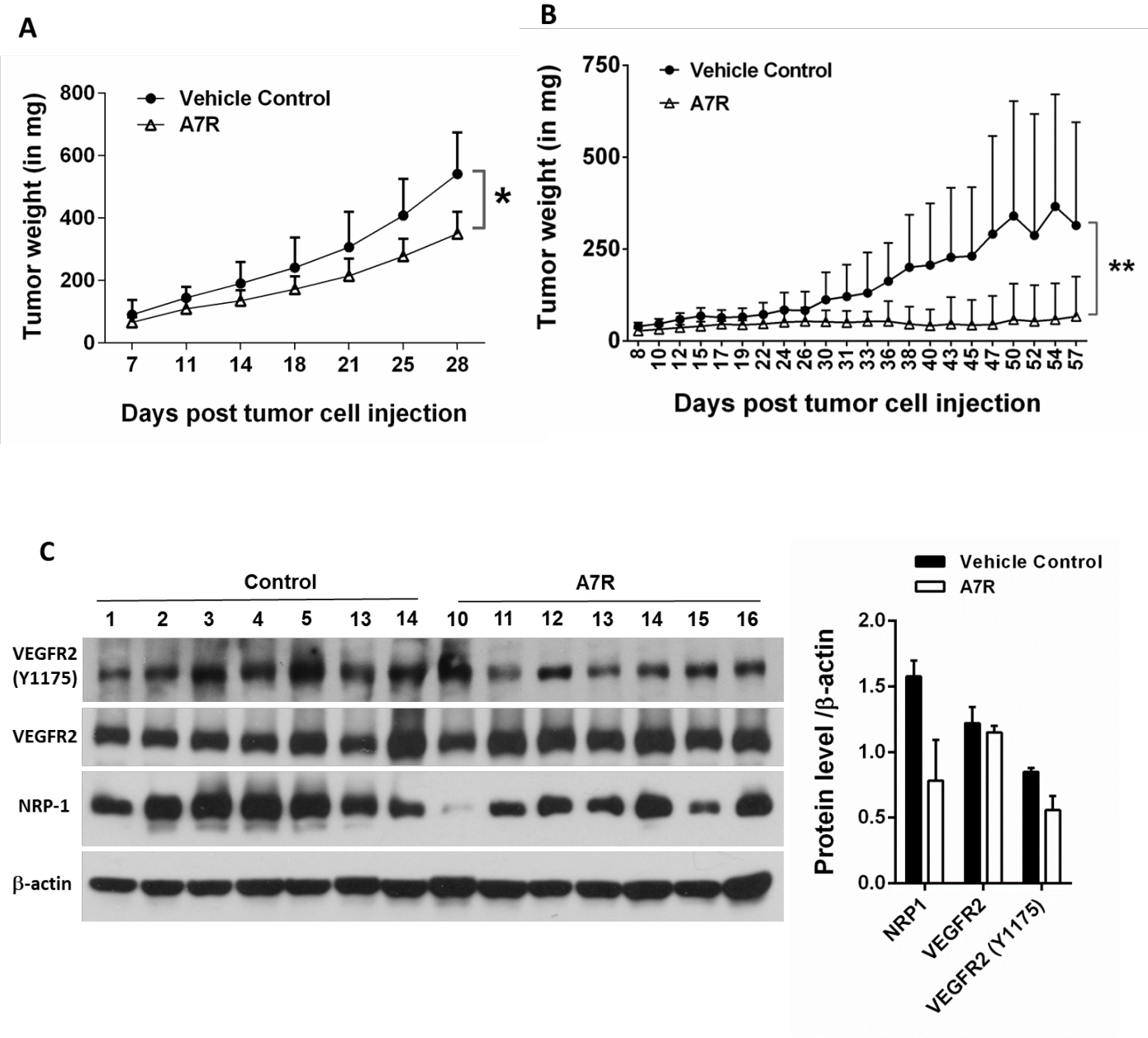


Figure 11: Blockade of NRP1 signaling attenuates MUC1hi tumor growth in vivo. A) A7R moderately attenuates KC tumor growth in C57BL/6 mice. **B)** A7R prevents BxPC3.MUC1 tumor growth in nude mice. **C)** A7R treatment in vivo reduces VEGFR2 phosphorylation as well as NRP1 level. Left, representative images; right, normalized protein expression level.



Task 3:

To directly target angiogenesis within the tumor microenvironment by using TAB004 conjugated to an inhibitory peptide that blocks VEGF-NRP1 interaction (12-36 months). In this aim, we will attempt targeted drug delivery using an antibody specific for tumor-associated MUC1 (TAB004) conjugated to a peptide inhibitor of VEGF-NRP1 binding.

With regard to targeted drug delivery using the TAB004 MUC1 monoclonal antibody, we first showed that TAB004 when injected intraperitoneally and intravenously specifically reaches the tumor within 24 hours (Figure 12). With iv injection, we observed some reaching the spleen. Therefore, for our future experiments, we plan to inject the antibody intraperitoneally. Using a pancreas cancer tissue array, MUC1 expression was detected in all PDA tissues restricted to the ductal epithelia, but the intensity of expression varied (Figure 13A). Similarly, NRP1 was expressed at varying levels in the tumors from the same 40 PDA patients (Figure 13B). Closely but not exactly, we found the higher the MUC1 expression in PDA, the stronger the NRP1 expression (Figure 13C). A nonparametric Spearman correlation of 0.70 was achieved with $n=65$ tissue-cores which was highly significant ($P < 0.0001$) and indicated a positive association between the MUC1 and NRP1 in human PDA. This finding further supports the hypothesis of blocking VEGF-NRP1 signaling with conjugate for treating MUC1hiNRP1hi PDA tumors.

Next, we showed by *in vitro* confocal microscopy that TAB004 was internalized into KCM cells through endocytosis (Figure 14). We have already shown that the blocking peptide alone is effective *in vivo* and that targeting antibody TAB004 alone internalizes in the cells and reaches the tumor bed *in vivo*. We have optimized and conjugated the NRP1 antagonist peptide/blocking peptide A7R to the antibody TAB004. We showed that TAB004 conjugated with NRP1 inhibitory peptide A7R (TAB004-A7R) can effectively reduce the VEGF production (Figure 15), with A7R concentration at approximately 0.5uM, much lower than the effective dose by A7R alone (~500uM). In addition, we have been working on the live imaging of MUC1-tumor bearing mice for tracking the specificity and effectiveness of TAB004 tumor targeting *in vivo* (Figure 16). We show that the TAB004 specifically localizes to the tumors sparing all normal organs.

It took us a while to get the optimal concentration of the TAB004-A7R conjugate that showed efficacy *in vitro* as shown in Figure 15. The mice with human and mouse tumors are developed and the treatment has been initiated. The results will be available in the next 2 months. We will send the results as soon as they are available. Given the *in vivo* efficacy of the NRP-1 inhibitor, we believe that direct targeting will further enhance the efficacy of the inhibitor. Most importantly, we show that the TAB004-NRP1 conjugate spares all normal organs and directly accumulates in the tumor as shown in Figure 16.

Figure 12: Localization of TAB004 in mice bearing the MUC1+ve tumor. Mice are injected with biotin-TAB004 (50ug/mouse) via the ip, it, and iv routes. After 24hrs, mice are euthanized and TAB004 binding is detected by IHC probing with streptavidin-HRP. Brown staining indicates MUC1 expression.

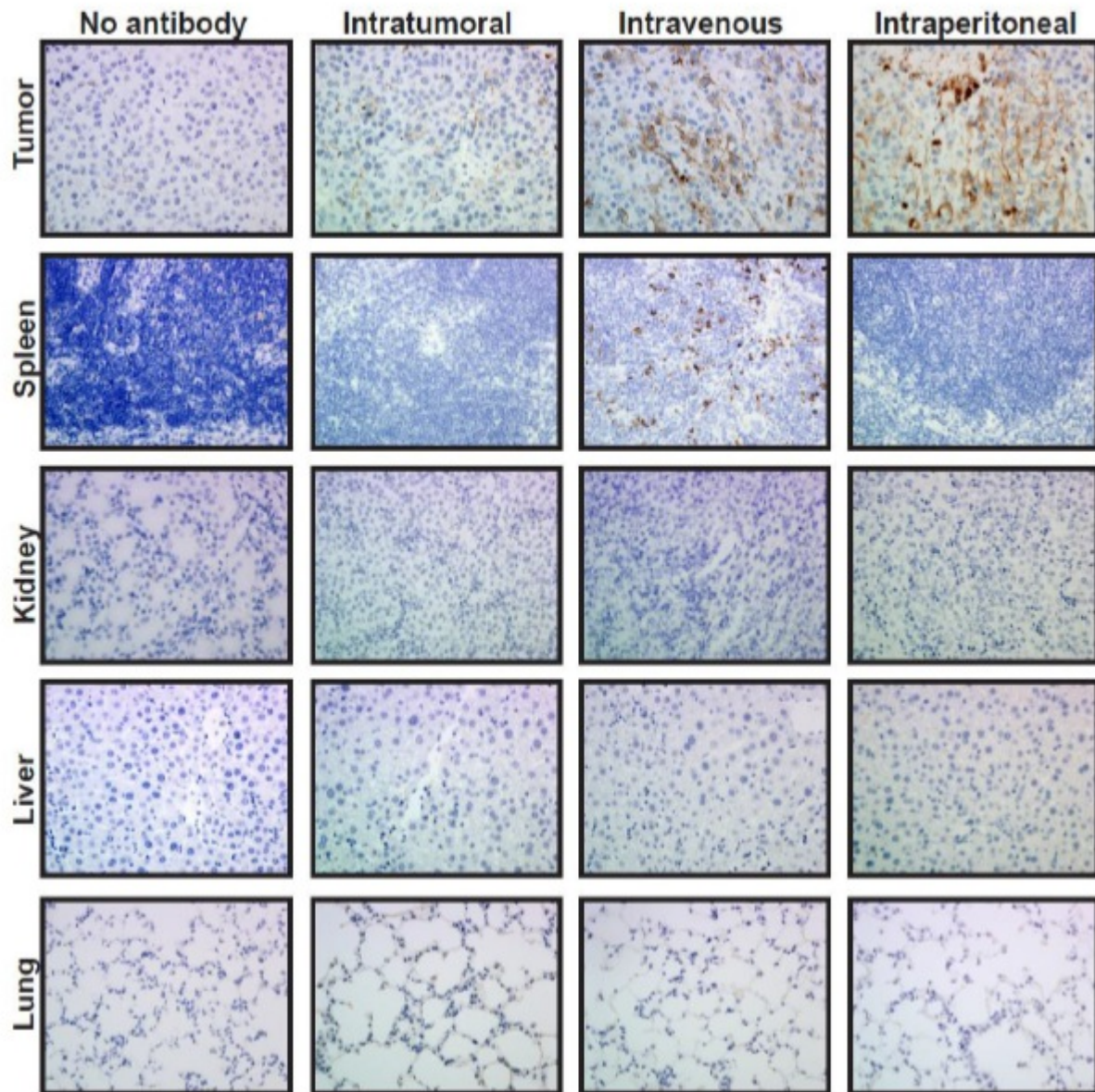
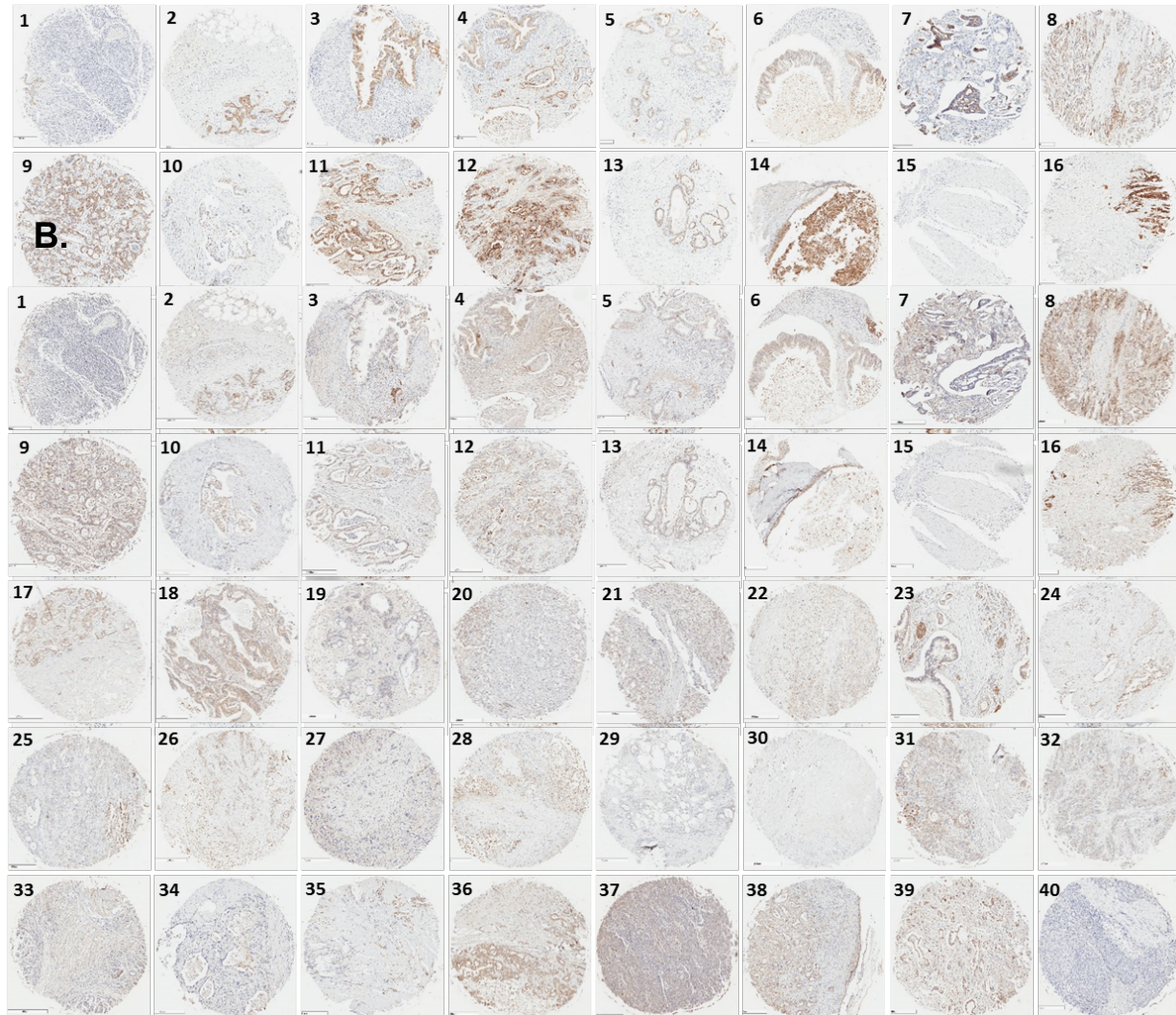


Figure 13: High correlation between the MUC1 and NRP1 expression in primary human PDA tumors. Pancreas tissue microarray (TMA) for expression of **A)** tumor form of MUC1 detected by TAB004 and **B)** NRP1. N=40 cores. **C)** A positive association between MUC1 level and NRP1 level as evidenced by a nonparametric Spearman correlation analysis. Black bar, MUC1 scores; yellow bar, NRP1 scores. The black bold arrow (\uparrow) points to the 4 cores out of forty in which the ratio for MUC1 vs NRP1 was >1.5 or <0.5 .

A.



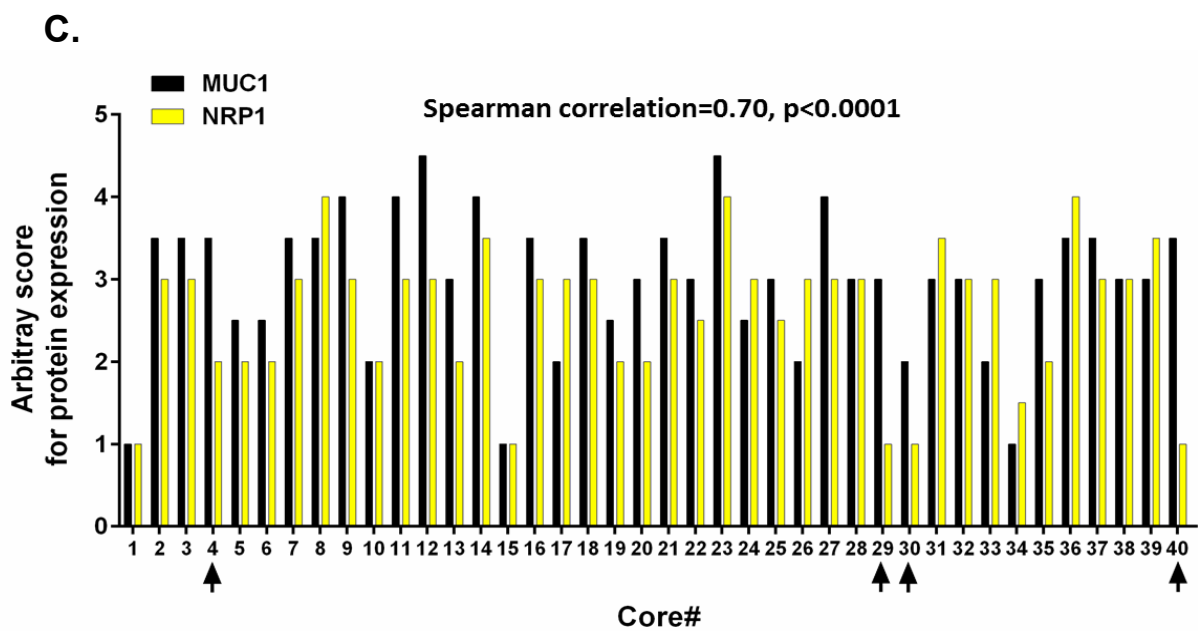


Figure 14: TAB004 binds MUC1 and get internalized in human MUC1-expressing KCM cells. KCM cells are plated on chamber tissue culture slides. Cell surface is labeled with Cell

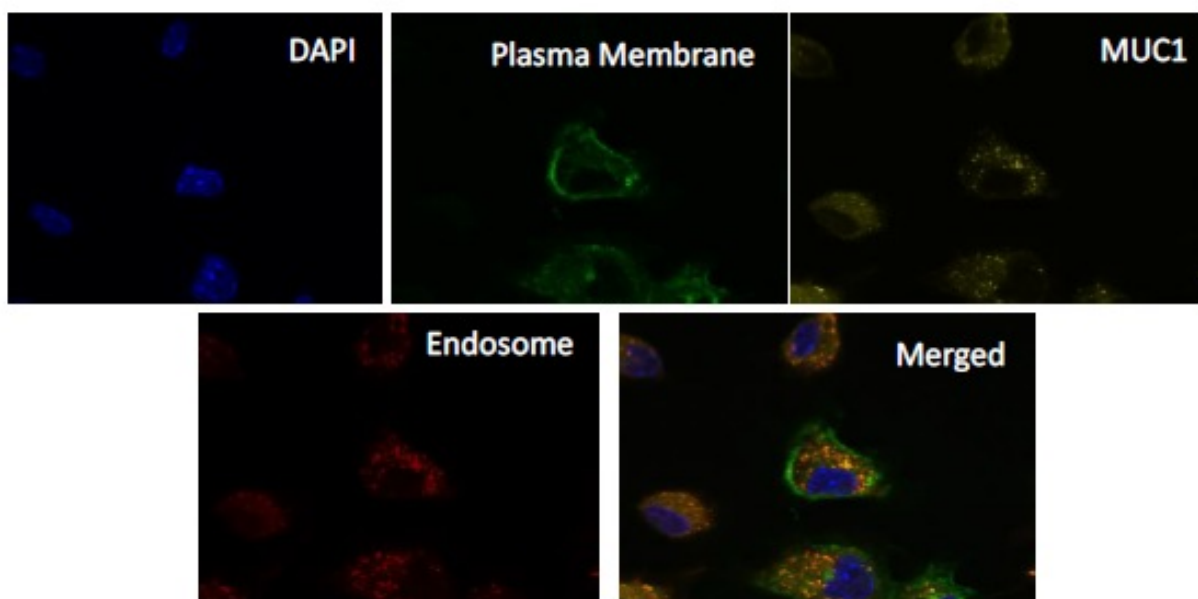


Figure 15: TAB004-A7R conjugate inhibits VEGF production in MUC1^{hi} HPAFII cells. A7R is conjugated with TAB004 at different peptide/antibody ratio (low=2.2; medium=4.1). HPAFII cells were treated as indicated for 24hr.

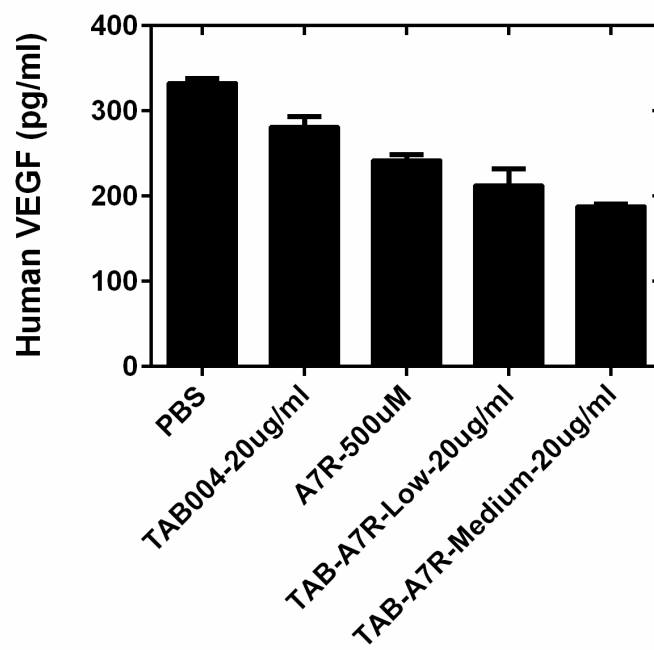
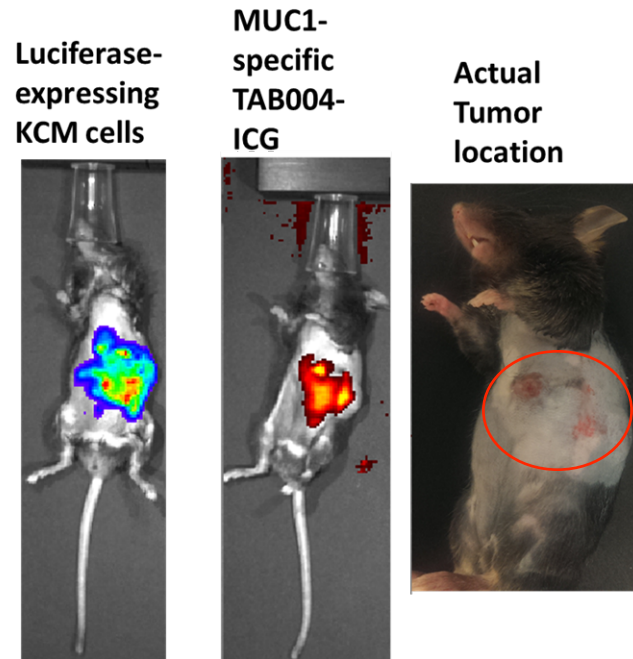


Figure 16: Lively tracking of TAB004 against MUC1-positive tumor in vivo. Luciferase-expressing KCM cells are orthotopically injected into pancreas of MUC1Tg C57BL/6mice. After 1-2 weeks, mice are intraperitoneally injected with TAB004-ICG. The signal of luciferase and TAB004-ICG are detected by IVIS imaging system.



Key Research Accomplishments

- MUC1 in pancreatic cancer cells regulate levels of NRP1, VEGFR and angiogenic signaling
- VEGFR, NRP1 and phosphorylation of VEGFR2 tyrosines are reduced in MUC1-ve tumors
- MUC1+ve PC cells regulate their own VEGF production autocrine manner and thus affect survival
- MUC1+ve PC cells promote endothelial cell tube formation in a NRP1-dependent fashion
- MUC1 up-regulates NRP1 and creates a pro-angiogenic niche in vivo
- MUC1+ve tumors respond to NRP1 blocking peptide but MUC1-ve cells do not. This suggests that MUC1 could be a companion diagnostic for anti-angiogenic therapy targeting NRP1.
- MUC1 antibody (TAB004) internalizes in the MUC1+ve tumor cells through endocytosis suggesting that the drug will be internalized when conjugated to TAB004.
- Established the four color confocal images for plasma membrane, lysosomes, MUC1, and DAPI
- TAB004 localizes to the tumor when injected intraperitoneal and intravenous within 24hours and spares normal organs
- MUC1 is specifically detected by TAB004 in primary human PDA tumors. Strong association between MUC1 and NRP1 is demonstrated. Findings strongly support the feasibility for in vivo treatment of MUC1+ PDA tumors with TAB004-A7R conjugate.
- TAB004-A7R conjugate inhibits VEGF production at very low dose *in vitro*.
- Live imaging of tumor bearing mice verify the MUC1-specific tumor targeting by TAB004, which will benefit the tracking of tumor growth in live animals over the study period.

Conclusion

In conclusion, we show that NRP1 may be an excellent target for treating MUC1+ve PDA but not MUC1-ve PDA. It is doubtful that a monotherapy with NRP1 blockade will work and therefore using the TAB004-conjugated to the drug may show promise due to target-specificity as well as ADCC caused by the antibody and thereby making the tumors more vulnerable to NRP1 blockade. In the long term, combination therapy with standard of care drug and NRP1 blockade conjugated to TAB004 or two drugs packaged in nanoparticles and conjugated to the antibody might be highly efficacious for patients with pancreatic cancer.

Publications, Abstracts, and Presentations

Ru Zhou, Jennifer M Curry, Lopamudra Das Roy, Priyanka Grover, Jamil Haider, Laura J. Moore, Shu-ta Wu, Anishaa Kamesh, Mahboubah Yazdanifar, William A. Ahrens, TinChung Leung, Pinku Mukherjee. A Novel Association of Neuropilin-1 and MUC1 in Pancreatic Ductal Adenocarcinoma: Role in Induction of VEGF Signaling and Angiogenesis. *Oncogene* 2015 (In press)

Ru Zhou, Jennifer Curry, Priyanka Grover, Lopamudra Das Roy, TinChung Leung, and Pinku Mukherjee. **MUC1 enhances neuropilin-1 signaling in pancreatic ductal adenocarcinoma.** AACR Annual Meeting 2014; April 5-9, 2014; San Diego, CA

Inventions, Patents and Licenses

None

Reportable Outcomes

- Publication in press in *Oncogene*. The other manuscript is in preparation for targeted therapy with TAB004-A7R conjugate in MUC1+ve PDA tumors.
- Research work has been presented as abstract in 2014 AACR annual meeting.
- Research Associate hired for the project and now promoted to Research Assistant Professor. Graduate and undergraduate students are working on parts of the project and are getting publications and degrees out of this work.
- Patient pancreatic cancer serum and tissue collected for future analysis.
- Primary human PDA tissue microarray has been well adapted in the lab due to this project, and can broaden the significance of research findings.
- KC, KCM, and KCKO cells are further characterized due to this project and is made available to other researchers
- Development of a novel antibody-anti-angiogenic drug conjugate that can be used in immune competent mouse model of spontaneous PDA and can be potentially translated to human clinical trials.

Other Achievements

References

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Appendices

None